

PhD thesis

**Investigation of the role of capsaicin-sensitive afferents, the
TRPV1 and the somatostatin receptor subtype 4 (sst₄) receptors in
murine airway inflammation models**

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I. INTRODUCTION

Our Neuropharmacology Research Group has investigated the role of capsaicin-sensitive sensory nerves in nociceptive and inflammatory conditions for decades. Several lines of evidence have been provided for the complex regulatory function of these fibres in skin and joint inflammation models, as well as inflammatory and neuropathic pain processes (Szolcsányi, 2004). Since previous data have revealed that afferent nerves and sensory neuropeptides released from them upon activation are involved in the pathogenesis of airway inflammation, my main PhD task was to develop *in vivo* murine models of pulmonary inflammation on the basis of the international literature with the help of which systematic experiments can be performed.

The airways are densely innervated by capsaicin-sensitive sensory nerves, which play an important regulatory role in inflammatory processes via the release of sensory neuropeptides. The Transient Receptor Potential Vanilloid 1 (TRPV1) receptor, also known as capsaicin receptor, is a non-selective cation channel expressed selectively in the cell membrane of thin afferent (C and A δ) fibres, which is activated/sensitized by noxious heat and a variety of inflammatory mediators, such as protons, lipoxygenase products, bradykinin or prostaglandins (Tominaga et al., 1998). Several results indicate that sensory innervation of the airways by capsaicin-sensitive peptidergic afferents is not only involved in sensory input, but neuropeptides released from them directly exert local bronchoconstrictor (Szolcsányi and Barthó, 1982) and inflammatory effector functions (Lundberg, 1995; Maggi, 1995; Szolcsányi, 1996). The pivotal role of this neurogenic mechanism was clearly shown in various inflammatory reactions evoked by a large scale of irritants, such as cigarette smoke, SO₂, ether, etc. (Barnes, 2001; Lundberg, 1995; McDonald, 1987). However, their role was found to be controversial when it was analyzed in longer-lasting pneumonia or asthma models using tachykinin receptor antagonists or pretreatment with large doses of capsaicin to destroy capsaicin-sensitive nerve endings. Pretreating rodents with high systemic doses of capsaicin or resiniferatoxin (RTX) induces degeneration of these capsaicin-sensitive C and A δ fibre chemonociceptive pulmonary nerve terminals making these fibres unresponsive to chemical stimuli without influencing their sensitivity to electrical stimuli (Bevan and Szolcsányi, 1990). It has long been described that tachykinins, such as substance P (SP) and neurokinin A (NKA), released from capsaicin-sensitive sensory nerve endings of the lung elicit neurogenic inflammation (vasodilatation and increased vascular permeability) in the airways of rats and guinea pigs and tachykinin-induced plasma protein extravasation is enhanced by the

concomitant release of calcitonin gene-related peptide (CGRP). Two tachykinin receptor subtypes, the neurokinin 1 (NK1) and neurokinin 2 (NK2) receptors mediate the biological actions of tachykinins in the airways, the preferred ligands for these receptors are SP and NKA, respectively. NK2 receptors have already been demonstrated to be involved in bronchoconstriction and bronchial hyperresponsiveness in several rat and guinea pig models and human studies, whereas NK1 receptors have been found to participate in neurogenic inflammation (Advenier et al, 1997; Joos et al, 2001; Lagente and Advenier, 1998). Contradictory data have been published in the airways on the effects of CGRP mediated predominantly via CGRP1 receptor activation. CGRP-induced bronchoconstriction in humans, as well as bronchodilatation in rats have been described.

More recently a surprising counter-regulatory humoral function of capsaicin-sensitive sensory nerve endings has been described. Several lines of evidence indicated that somatostatin released from these terminals reaches the circulation and elicits systemic anti-inflammatory and analgesic “sensocrine” effects (Szolcsányi et al, 2004). These systemic inhibitory actions of somatostatin evoked e.g. by antidromic stimulation of the rat sciatic nerve inhibited plasma protein extravasation in the trachea and the mediastinal connective tissue. Furthermore, bilateral stimulation of the peripheral stumps of the cut vagal nerves in rats pretreated with atropine or hexamethonium inhibited neurogenic inflammation in the rat hindpaw skin besides inducing neurogenic inflammation locally in the trachea.

Somatostatin in 14 and 28 amino acid-containing forms is widely distributed throughout the body. It exerts a wide range of effects such as modulation of hormone and neurotransmitter release, cognitive and behavioral processes, the gastrointestinal tract, the cardiovascular system and tumor cell proliferation. These effects are mediated via five different G-protein associated somatostatin receptor subtypes (sst₁-sst₅) which can be divided into two main groups on the basis of their sequence similarities and their binding profile towards synthetic somatostatin analogues: the SRIF₁ group comprises the sst₂, sst₃ and sst₅ receptors and the SRIF₂ group contains the sst₁ and sst₄ receptors (Hoyer et al, 1995; Pintér et al, 2006). Several data indicate that receptors in the SRIF₁ group mediate the endocrine and anti-proliferative effects of somatostatin, while our previous data revealed that the SRIF₂ group is likely to be responsible for the anti-inflammatory and anti-nociceptive actions. Although these results suggested that the anti-inflammatory actions of somatostatin are mediated predominantly via sst₄ receptors, direct evidence could not be provided to support this idea due to the lack of sst₄-selective antagonists. Furthermore, as compared to other

receptor subtypes, there are relatively few data in literature available on the localization of sst₄ receptors (rev. Pintér et al, 2006; Szolcsányi et al, 2004).

Although native somatostatin is not a suitable candidate for the development of anti-inflammatory drugs due to its short half-life and wide range of effects, stable, sst₄-selective agonists could be promising. TT-232, a stable cyclic heptapeptide analog, synthesized by the Peptide Chemistry Research Group of the Hungarian Academy of Sciences (Department of Medical Chemistry, Semmelweis University, Budapest) showed the highest affinity to sst₄ receptors (Helyes et al, 2006). This compound failed to inhibit growth hormone release or gastrin secretion, but it had strong anti-proliferative, anti-inflammatory and anti-nociceptive effects in several experimental models (rev. Helyes et al, 2006). A novel sulfonamido-peptidomimetic compound, J-2156, synthesized at Juvantia Pharma (Turku, Finland), belongs to a chemically novel class of somatostatin receptor ligands. J-2156 possesses nanomolar affinity for the human sst₄ and it is 400-fold more selective for this receptor than any other human sst receptor subtype. J-2156 exerted greater agonism on the sst₄ than its endogenous ligands and it has been defined as a selective, high affinity agonist (Engstrom et al, 2005).

II. AIMS OF THE STUDY

1. Investigating the role of capsaicin-sensitive fibres and sensory neuropeptides in endotoxin-induced airway inflammation and consequent bronchial hyperreactivity with functional, morphological and biochemical techniques in mice.
2. Analysing the function of TRPV1 capsaicin receptors in endotoxin-induced pulmonary inflammation and related bronchial hyperresponsiveness using receptor gene-deficient mice also with functional, morphological and biochemical techniques.
3. Immunohistochemical and molecular biological examination of the somatostatin sst₄ receptor protein and mRNA expression in the mouse lung and its inflammation-induced alterations. Integrative investigation of the function of sst₄ receptors in the endotoxin-evoked inflammatory reaction and consequent bronchial hyperreactivity using receptor gene-deleted mice.
4. Examination of the effects the sst₄/sst₁ receptor agonist heptapeptide TT-232 and the highly sst₄-selective peptidomimetic agonist J-2156 in acute and chronic airway inflammation models.

III. METHODS

Animals

Experiments were performed on female CD1, Balb/c and C57BL/6 mice weighing 20-25 g. Some studies were performed on two lines of receptor gene knockout mice (TRPV1^{-/-} obtained from Dr. J.B. Davis, GlaxoSmithKline, Harlow, U.K.; sst4^{-/-} kindly provided by Dr. Piers Emson Laboratory of Molecular Neuroscience, The Babraham Institute, Cambridge, U.K.) and their wild-type counterparts (TRPV1^{+/+}, sst4^{+/+}).

Endotoxin-induced airway inflammation model

Endotoxins are constituents of the outer layer of gram negative bacteria, they can be found in the surrounding microenvironment, it is a significant risk factor for asthma and asthma severity depends to a great extent on its concentration. Intranasal administration of lipopolysaccharide (LPS), the main component of endotoxins, to mice is a commonly used confined experimental model to study acute lung inflammation without causing systemic multiple organ dysfunction. Intranasal LPS induces a huge influx of neutrophils, consequent extravasation of plasma proteins into the airways leading to perivascular/peribronchial oedema formation and activation of macrophages. Therefore, subacute airway inflammation was evoked by intranasal application of *Escherichia coli* LPS (serotype; 60 µl, 167 µg/ml) under light ether anaesthesia 24 h prior to the airway reactivity measurements.

Ovalbumin-induced asthma model

In the ovalbumin-induced chronic lung inflammation model of Balb/c mice sensitization was induced by i.p. injection of ovalbumin (OVA; 20 µg/mouse) on days 1 and 14, then local inflammation in the airways was evoked by inhalation of 5% OVA solution on days 28, 29 and 30. Airway reactivity measurement was performed with whole body plethysmography on day 32.

Treatments, pretreatments

In the first series of experiments capsaicin-sensitive nerve endings were inactivated by pretreating the mice with repeated high doses of the TRPV1 receptor agonist resiniferatoxin (RTX; 30, 70 and 100 µg/kg RTX s.c. on 3 consecutive days 14 days before LPS). RTX was dissolved in absolute ethanol to make a 1 mg/ml stock solution and further diluted with saline. To examine the involvement of pro-inflammatory sensory neuropeptides in the development of airway inflammation, other groups of animals were treated with the NK1 receptor antagonist SR 140333 (160 µg/kg s.c.), the NK₂ receptor antagonist SR 48968 (160 µg/kg

s.c.), their combination or the CGRP1 receptor antagonist CGRP(8-37) (160 µg/kg s.c.) 30 min before, 8 h and 23 h after LPS administration.

In the second series of studies a group of TRPV1^{+/+} mice was treated with the somatostatin receptor antagonist cyclo-somatostatin (C-SOM; 250 µg/kg i.p.) for examining the functional roles of somatostatin released via TRPV1 activation in LPS-induced airway inflammation. To investigate the effect of exogenous somatostatin on the inflammatory response, somatostatin-14 (SOM-14; 100 µg/kg i.p.) was administered in both TRPV1^{-/-} and TRPV1^{+/+} animals. SOM-14 and C-SOM were dissolved freshly in saline and injected 30 min before LPS instillation and every 6 h during the 24 h experimental period.

In the fourth set of experiments two sst₄ receptor agonists, TT-232 and J-2156 were injected i.p. (500 µg/kg) repeatedly during the experiments to study their effects on the development of the inflammatory reactions. In the LPS model they were administered 3 times during the 24 h of the study (20 minutes before, 12 and 24 h after the induction of the inflammation). In the OVA model the compounds were injected i.p. on days 28, 29 and 30, twenty minutes before each OVA inhalation, 6 h and 12 h afterwards. An additional dose was injected 20 minutes before the measurement. In separate groups a single 500 µg/kg dose of these agents was injected i.p. 20 min before plethysmography measurements after the development of the inflammation, to examine their effects on airway hyperreactivity without influencing the inflammatory reactions.

Assessment of bronchoconstriction

Airway responsiveness in conscious, spontaneously breathing animals was measured by recording respiratory pressure curves by whole body plethysmography (Buxco Europe Ltd, UK) 24 h after LPS administration in the acute model and 31 days after the first i.p. OVA injection in the chronic model. Aerosolized saline and then the muscarinic acetylcholine receptor agonist carbachol (carbamoyl-choline) in increasing concentrations (50 µl per mouse for 1.5 min in 5.5, 11 and 22 mM concentrations) were nebulized through an inlet of the main chamber for 50 sec to induce bronchoconstriction and readings were taken and averaged for 15 minutes following each nebulization. Enhanced pause (Penh) was measured as an indicator of bronchoconstriction and consequent increase of airway resistance. Penh is a complex, calculated parameter ((expiratory time/ relaxation time)-1): (max. expiratory flow/ max. inspiratory flow).

Histological studies and scoring

At the end of the experiments the animals were killed, the lungs were excised and put into formaldehyde for histological processing and stained with hematoxylin and eosin or periodic acid-Schiff (PAS) to more precisely visualize mucus producing goblet cells. Semiquantitative scoring of the inflammatory changes was performed by an expert pathologist blinded from the study.

Determination of sensory neuropeptides with radioimmunoassay

Frozen lung samples were thawed, chopped into small pieces, homogenized and centrifuged. The pellet was used for myeloperoxidase assay (see below) and the supernatant was removed for direct radioimmunoassay (RIA) measurements of SP, CGRP and somatostatin. Plasma somatostatin concentration was determined from arterial blood samples.

Measurement of myeloperoxidase (MPO) activity and inflammatory cytokine concentrations in the lung

Accumulation of granulocytes, especially neutrophils, was measured from homogenized lung samples by assessment of MPO activity. Neutrophil accumulation was determined by comparing MPO enzyme activity of the samples to a human standard MPO preparation. Reactions were performed in 96-well microtitre plates in room temperature. The concentration of the inflammatory cytokine IL-1 β , TNF- α was determined by specific ELISA technique (BD Sciences Eastern Europe).

Detection of the sst₄ receptor protein in the lung

Lung specimens were fixed in formaldehyde for 24 h, embedded in paraffin and 5-7 μ m sections were made with a microtome. The antigen was revealed by incubating the slides in acidic (pH 6) citrate-buffer in the microwave. The slides were incubated with 1:50 dilution of rabbit polyclonal anti-sst₄ antibody, then with horseradish peroxidase (HRP)-conjugated En Vision system anti-rabbit secondary antibody. Finally, immunolocalization of the sst₄ receptor was detected by diaminobenzidine (DAB) development.

Determination of sst₄ receptor mRNA in the lung

Lung samples were stored in 1 ml RNAlater solution at -80°C for further processing. Total RNA was isolated using GenElute Mammalian Total RNA Miniprep Kit with proteinase K, according to the manufacturer's instructions. Specific mRNA levels were quantified by the "LightCycler RNA Master SYBR Green I" (Roche) quantitative real-time reverse transcriptase-polymerase chain reaction RT-PCR assay on a LightCycler system.

IV. RESULTS

IV.1. ROLE OF CAPSAICIN-SENSITIVE AFFERENTS AND SENSORY NEUROPEPTIDES IN ENDOTOXIN-INDUCED AIRWAY INFLAMMATION AND CONSEQUENT BRONCHIAL HYPERREACTIVITY IN THE MOUSE (Elekes et al, Reg. Pept. 14: 44-54, 2007.)

Endotoxin-induced alterations of SP and CGRP concentrations in the lung

SP and CGRP concentrations in the lung increased by 28% and 30%, respectively, 25 h after intranasal administration of LPS. Basal peptide contents of the lung of RTX-pretreated mice did not differ significantly from the values of untreated mice. Impairment of the function of capsaicin-sensitive sensory fibres with RTX desensitization prevented the endotoxin-induced elevation of both peptides pointing out that capsaicin-sensitive nerve terminals are the source of LPS-evoked increase of SP and CGRP concentrations.

Inflammatory morphological changes in the lung

Histological examination and scoring revealed that compared to the intact, uninflamed lung structure, LPS induced marked peribronchial/perivascular oedema formation, granulocyte accumulation around the bronchi, infiltration of mononuclear cells (mainly macrophages) into the alveolar spaces and hyperplasia of mucus producing goblet cells. The composite inflammatory score calculated from these parameters was significantly greater in mice pretreated with RTX. Comparison of the score values separately for the four parameters in cases of the LPS-treated and RTX-desensitized LPS-treated groups revealed that although RTX desensitization did not alter the extent of oedema and macrophage infiltration, the number of goblet cells and peribronchial granulocytes were significantly greater in RTX-pretreated animals. The NK1 receptor antagonist SR 140333 or the NK2 receptor antagonist SR 48968 had no effect on any endotoxin-evoked inflammatory parameters of the composite inflammatory score. However, their combination or the CGRP1 receptor antagonist CGRP(8-37) reduced the number of granulocytes accumulated around the bronchi. On the contrary, neither treatments influenced oedema formation, the number of mononuclear cells in the alveolar spaces and goblet cell hyperplasia, therefore, no difference could be detected in the composite inflammatory scores. These data indicate that in the LPS-induced airway inflammation model neurogenic inflammatory mediators, such as tachykinins or CGRP acting at NK1, NK2 or CGRP1 receptors, respectively, do not play a significant role in the overall histological changes one day after the challenge.

Inflammatory airway hyperreactivity

Penh significantly increased after intranasal LPS treatment compared to the untreated group. Inhalation of increasing concentrations (5.5 - 22 mM) of the muscarinic receptor agonist carbachol evoked a concentration-dependent bronchoconstriction shown by the Penh curves. In mice pretreated with RTX the LPS-induced airway hyperreactivity was completely absent at the highest carbachol concentration, while it was inhibited by 70-85% at lower concentrations of the cholinergic agonist. The LPS-evoked airway hyperreactivity remained practically unchanged after treating the mice with the NK1 receptor antagonist SR 140333 or the CGRP1 receptor antagonist CGRP(8-37). However, the NK2 antagonist SR 48968 induced a significant inhibition, which remained about the same in case of combined treatment with the two tachykinin receptor antagonists.

Endotoxin-induced increase of lung and plasma somatostatin concentrations

In non-desensitized mice intranasal LPS administration induced about two-fold elevations of both lung and plasma somatostatin levels, which was prevented after the destruction of capsaicin-sensitive afferents by RTX pretreatment. The basal somatostatin content was also slightly decreased after RTX desensitization, but the difference did not prove to be significant.

IV.2. ROLE OF TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 RECEPTORS IN ENDOTOXIN-INDUCED AIRWAY INFLAMMATION IN THE MOUSE (Helyes et al Am. J. Physiol. Lung Cell. Mol. Physiol. **292: L1173-1181, 2007.)**

Inflammatory airway hyperresponsiveness in TRPV1^{+/+} and TRPV1^{-/-} mice

Responses demonstrated as percentage increase of Penh was markedly enhanced in the LPS-treated groups compared to the respective non-inflamed controls pointing out the development of inflammatory bronchial hyperresponsiveness. In response to LPS-induced inflammation both maximal Penh values were higher and the duration of the bronchoconstriction was prolonged in mice lacking the TRPV1 receptor.

Inflammatory changes in the lung of TRPV1^{+/+} and TRPV1^{-/-} mice

Histological examination and scoring revealed that compared to the intact lung structure LPS induced marked peribronchial/perivascular oedema formation, granulocyte accumulation around the bronchi, infiltration of mononuclear cells and hyperplasia of mucus producing goblet cells. All these inflammatory parameters were significantly more severe in TRPV1 receptor gene-deficient mice than in their wildtype counterparts.

Myeloperoxidase activity in the lung of TRPV1^{+/+} and TRPV1^{-/-} mice

Endotoxin administration induced about 2-fold and 4-fold elevations of MPO activity in the lung of TRPV1^{+/+} and TRPV1^{-/-} mice, respectively. This quantitative marker of accumulated granulocytes in the inflamed tissue was significantly greater, more than double in the TRPV1 receptor knockout group.

Somatostatin concentration in the lung and plasma

The basal level of somatostatin-like immunoreactivity was about 4-5 folds higher in the lung than in the plasma of both wildtype and TRPV1 gene-deleted animals without a significant difference between the two groups. In response to LPS administration there was a pronounced increase of somatostatin-like immunoreactivity in the lung and plasma of TRPV1^{+/+} mice, while in the TRPV1^{-/-} group the LPS-induced elevation of somatostatin level was much smaller in the lung and absent in the plasma. These results point out a TRPV1 receptor-mediated release of this neuropeptide from the sensory fibres of the lung, which gets into the systemic circulation.

Role of somatostatin in endotoxin-evoked airway hyperresponsiveness, inflammatory histopathological changes and myeloperoxidase activity

Repeated treatments of TRPV1^{-/-} with SOM-14 (100 µg/kg i.p.) markedly inhibited bronchoconstriction induced by 11, 22 and 44 mM carbachol. Similarly, the same doses of somatostatin also significantly diminished inflammatory airway hyperreactivity in TRPV1^{+/+} mice. Meanwhile, cyclo-somatostatin (250 µg/kg i.p.) significantly enhanced bronchial hyperreactivity in TRPV1^{+/+} mice. Somatostatin administration diminished endotoxin-induced inflammatory changes both in KO mice and their wildtype counterparts. C-SOM injection in the TRPV1^{+/+} group aggravated these parameters, especially peribronchial oedema formation, granulocyte infiltration and goblet cell hyperplasia. In accordance with these histological findings somatostatin significantly decreased LPS-evoked MPO activity in the lung of TRPV1^{-/-} and TRPV1^{+/+} mice. Administration of the antagonist induced a more than 2-fold elevation in the TRPV1^{+/+} group. No change was observed in carbachol-induced bronchoconstriction, histological parameters and MPO activity after repeated administration

of the same doses of the antagonist, as well as SOM-14 (100 µg/kg i.p., 4 times) to intact TRPV1^{+/+} and TRPV1^{-/-} mice as compared to the respective intact animals.

IV.3. EXPRESSION OF SOMATOSTATIN RECEPTOR SUBTYPE 4 (SST₄) IN THE MOUSE LUNG AND ITS ROLE IN ENDOTOXIN-INDUCED AIRWAY INFLAMMATION

Expression of sst₄ receptor in the intact and inflamed lung

Immunolocalization of the sst₄ receptor protein in the intact lung was clearly detected on the luminal surface of the bronchial epithelial cells. Endotoxin induced a marked infiltration of neutrophils and mononuclear cells predominantly into the peribronchial/perivascular spaces 24 h after its intranasal administration pointing out the development of an acute interstitial pneumonitis. The accumulating mononuclear cells showed remarkable sst₄ positivity, while neutrophils seemed not to express this receptor. Molecular biological results showed that sst₄ receptor mRNA was present in the lung, but its concentration compared to the housekeeping β2-mikroglobulin mRNA level was not altered under inflammatory conditions in the endotoxin-induced pneumonitis model.

Inflammatory airway hyperresponsiveness in sst₄^{+/+} and sst₄^{-/-} mice

In response to LPS-induced inflammation both maximal Penh values were higher and the duration of the bronchoconstriction was prolonged in mice lacking the sst₄ receptor.

Inflammatory changes in the lung of sst₄^{+/+} and sst₄^{-/-} mice

Histological examination and scoring revealed that compared to the intact lung structure LPS induced marked peribronchial/perivascular oedema formation, granulocyte accumulation around the bronchi, infiltration of mononuclear cells and hyperplasia of mucus producing goblet cells. All these inflammatory parameters were significantly more severe in sst₄ receptor gene-deficient mice than in their wildtype counterparts.

Myeloperoxidase activity in the lung of sst₄^{+/+} and sst₄^{-/-} mice

Endotoxin administration induced significant elevations of MPO activity in the lung of both sst₄^{+/+} and sst₄^{-/-} mice as compared to their respective intact controls. This quantitative marker of accumulated granulocytes in the inflamed tissue was significantly, about 3 folds greater in the sst₄^{-/-} group than in the sst₄^{+/+} one.

Concentrations of inflammatory cytokines in the lung of $sst_4^{+/+}$ and $sst_4^{-/-}$ mice

LPS induced an about 32- and 12-fold increase of IL-1 β and TNF- α concentrations in the lung of $sst_4^{+/+}$ mice 25 h after the induction of the pulmonary inflammation. These elevations were significantly greater, almost double in the sst_4 gene-deleted group.

IV.4. ANTI-INFLAMMATORY AND ANTI-HYPERREACTIVITY EFFECTS OF SYNTHETIC SOMATOSTATIN RECEPTOR SUBTYPE 4 AGONISTS IN MURINE AIRWAY INFLAMMATION MODELS (Elekes et al, Eur. J. Pharmacol. <http://dx.doi.org/10.1016/j.ejphar>, 2007.)

Effect of TT-232 and J-2156 on endotoxin-induced inflammatory airway hyperreactivity

Inhalation of increasing concentrations (5.5 - 22 mM) of the muscarinic receptor agonist carbachol evoked a concentration-dependent bronchoconstriction shown by the Penh curves. J-2156, as well as TT-232 (500 μ g/kg i.p.) significantly inhibited endotoxin-induced airway hyperreactivity to a similar extent, both after a single acute administration and after repeated injections, although the degree of the inhibition was greater when the compound was given 3 times throughout the 24 h-experimental period. This result shows that these agonists are able to diminish carbachol-evoked bronchoconstriction via a direct action on bronchial smooth muscle cells besides inhibiting the development of the inflammation.

Effect of TT-232 and J-2156 on ovalbumin-evoked inflammatory airway hyperreactivity

OVA inhalation in sensitized animals markedly increased airway responsiveness to increasing concentrations (5.5 - 22 mM) of carbachol. Both TT-232 and J-2156 induced an about 50% inhibitory action on bronchoconstriction after administration of a single 500 μ g/kg i.p. dose. The extent of the effect observed in case of repeated (3x3) J-2156 injections was very similar. However, when TT-232 was injected repeatedly throughout the development of the inflammatory reaction airway hyperreactivity was abolished and carbachol-induced bronchoconstriction was even smaller than in non-inflamed control mice.

Effect of TT-232 and J-2156 on endotoxin-induced acute airway inflammation

Histological examination and scoring revealed that compared to the intact lung, intranasal LPS administration induced peribronchial/perivascular oedema formation, granulocyte accumulation around the bronchi, infiltration of mononuclear cells (mainly macrophages) into the alveolar spaces and hyperplasia of mucus producing goblet cells. These inflammatory changes were markedly reduced after repeated (3 times during the 24h

experimental period) i.p. treatments with J-2165 or TT-232. Semiquantitative scoring of these LPS-induced histopathological changes revealed that the extent of perivascular oedema, granulocyte accumulation and goblet cell hyperplasia were significantly decreased in both the J-2156 and TT-232-treated groups. The fourth parameter, alveolar macrophage infiltration was also decreased, but the extent of inhibition did not prove to be significant.

Effect of TT-232 and J-2156 on ovalbumin-evoked chronic airway inflammation

Morphological examination of the lung samples in the chronic airway inflammation model showed that compared to the intact lung, infiltration of eosinophil cells, mucosal oedema formation, enhanced mucus production and destruction of the epithelial cells was observed in response to ovalbumin exposure in sensitized mice. Repeated administrations of TT-232 exerted a significant inhibitory action on all the three inflammatory parameters. Meanwhile, J-2156 markedly diminished eosinophil infiltration and epithelial cell damage, but failed to reduce mucosal oedema and mucus production.

V. DISCUSSION AND CONCLUSIONS

The present results provide the first evidence that during endotoxin-induced airway inflammation of the mouse neurogenic inflammatory mediators, such as SP and CGRP, are released from capsaicin-sensitive sensory nerve terminals. The increased concentrations of these peptides in the lung one day after intranasal LPS application was completely absent in mice in which the capsaicin-sensitive nerve endings were selectively destroyed by resiniferatoxin pretreatment. On the other hand, it is worth mentioning that basal SP and CGRP contents of the lung was not altered after RTX pretreatment indicating that there is a considerable amount of these peptides in other non-neural cells, such as airway epithelial cells (Hastings and Hua, 1995), pulmonary neuroendocrine cells and immune cells (Nelson and Bost, 2004), which is not depleted by RTX pretreatment (Szallási and Blumberg, 1989; Szolcsányi et al, 1990). A possible explanation for this surprising finding can be that in the uninflamed, intact lung the concentrations of these sensory neuropeptides in capsaicin-sensitive fibres is relatively small compared to their total amount. Under inflammatory conditions these nerve terminals are stimulated by inflammatory mediators (protons, leukotriens, prostaglandins, bradykinin, etc.), which increases the neuropeptide synthesis in the dorsal root ganglia, enhances their axonal transport towards the periphery and presumably increases their release (Baluk et al, 1999). Since in the intact lung the neural fraction of the total SP and CGRP content is negligible compared to the non-neural pool, the inflammation-

induced 30% elevation of neurally-derived peptide concentrations is in fact a very considerable increase. Impaired function of these fibres with RTX pretreatment increased inflammatory histological changes, MPO activity and the production of the inflammatory cytokine IL-1 β . These data indicate that the effects of the released pro-inflammatory sensory neuropeptides in LPS-evoked inflammation are counteracted by other anti-inflammatory mediator(s) released also from the C-fibres (Lundberg, 1995). Our study with respective NK1, NK2 and CGRP receptor antagonists revealed mediator roles of SP and CGRP in neutrophil accumulation measured by MPO activity and IL-1 β production.

In LPS-evoked murine airway inflammation functional roles of the released tachykinins and CGRP was revealed with the aid of their respective receptor antagonists, but only concerning granulocyte accumulation and the production of the inflammatory cytokine IL-1 β . Endotoxin-induced enhancement of lung MPO activity, as a quantitative biochemical marker of granulocyte accumulation, was abolished after treatment with a combination of NK1 and NK2 receptor antagonists or the CGRP1 receptor antagonist CGRP(8-37). Interestingly, the increased concentration of IL-1 β in the lung was also inhibited by these treatments, but in the case of combined NK1 and NK2 receptor antagonists the blockade was incomplete. It has recently been described that only the combination of the tachykinin NK1 and NK2 receptor antagonists resulted in a significant reduction of neutrophil influx in a similar LPS-induced mouse model of airway inflammation. Activation of NK1+NK2 receptors and CGRP1 receptors localized on the surface of endothelial cells and granulocytes is involved in the expression of adhesion molecules and consequent leukocyte adhesion and accumulation. Appropriate activity and synergistic interaction of both CGRP1 receptors and tachykinin receptors might be necessary for the granulocyte accumulation process and inhibition of one of them might be sufficient to block LPS-induced leukocyte recruitment. MPO activity and IL-1 β are very closely related inflammatory parameters, several data report that IL-1 β is potent in exerting neutrophil accumulation, but not oedema formation. Furthermore, granulocytes, besides many other inflammatory cells, are also able to synthesize IL-1 β . In accordance with our biochemical findings, neutrophil infiltration in histology was also inhibited by the CGRP1 receptor antagonist and the combination of the NK1 and NK2 receptor antagonists. However, none of the antagonists or their combination influenced significantly the composite inflammatory score, presumably because this value is calculated on the basis of four different characteristics among which leukocyte accumulation is only one parameter. The other three parameters, such as perivascular oedema formation, macrophage

infiltration and hyperplasia of mucus-producing goblet cells were not altered by any of the treatments. This finding suggests that these pro-inflammatory sensory neuropeptides play only a minor role in the overall severity of endotoxin-induced airway inflammation. In our model NK2 receptors play a predominant role in bronchial hyperreactivity, since the NK2 receptor antagonist SR 48968, unlike SR 140333 or CGRP(8-37) inhibited this response. This is in accordance with other data showing that NK2 tachykinin receptors are involved in bronchoconstriction and bronchial hyperresponsiveness in several other animal models and human studies.

We investigated the role of TRPV1 receptors localized selectively on capsaicin-sensitive fibres in endotoxin-induced airway inflammation and consequent bronchial hyperreactivity with functional, morphological and biochemical techniques using receptor gene-deficient mice. The inflammatory reaction as revealed by myeloperoxidase activity measurement and histological assessments, as well as consequent bronchial hyperreactivity were enhanced in TRPV1 gene-deleted mice compared to their wildtype counterparts. The released neuropeptides in turn influence the inflammatory process by acting at receptors localized on these peripheral nerve terminal themselves, vascular endothelial, bronchial epithelial and inflammatory cells. There is increasing evidence that neuropeptides are synthesized in non-neural sources as well, e.g. mononuclear cells and lymphocytes, and they can be released in a TRPV1-independent manner, especially under inflammatory conditions. Pro-inflammatory neuropeptides such as the previously discussed SP, neurokinin A and CGRP are localized in capsaicin-sensitive unmyelinated and thinly myelinated sensory fibres (C- and A δ fibres) in the airways of several species, their release from these nerve terminals and participation in the enhancement of both vascular and cellular phases of several inflammatory reactions is well established. Besides these pro-inflammatory neuropeptides, somatostatin is also present in the capsaicin-sensitive afferents of the lung (Hökfelt et al, 1976). However, immunohistochemical studies have provided evidence that somatostatin and substance P/CGRP are localized in distinct subpopulations of primary sensory neurones. Intranasal endotoxin administration increased somatostatin concentration in the lung and also in the plasma of wildtype mice. In the TRPV1 gene-deleted group LPS-induced pronounced rise of somatostatin concentration was abolished in the plasma and markedly inhibited in the lung. These results indicate that inflammatory mediators stimulate the TRPV1 receptor on sensory nerve terminals and elicit the release of somatostatin from these afferents. LPS-induced production of an “inflammatory mixture” containing lipoxygenase products, protons, prostaglandins, bradykinin, etc., which all activate/sensitize the integrative TRPV1 ion

channel localized on somatostatin-containing capsaicin-sensitive fibres in the airways, although even in this model some involvement of decreased tachykinin release cannot be ruled out in TRPV1^{-/-} mice. In LPS-treated TRPV1^{-/-} mice exogenous administration of somatostatin-14 prevented both increased bronchoconstriction and enhanced inflammatory reaction determined by MPO and histology. On the other hand, inhibitory function of the released somatostatin in the development airway inflammation and hyperresponsiveness was further evidenced in wildtype mice treated with the somatostatin receptor antagonist cyclo-somatostatin. This compound which inhibits the actions of somatostatin at all the five receptor subtypes (sst₁-sst₅) enhanced both inflammatory responses and bronchial hyperreactivity. Somatostatin effectively inhibits the release of pro-inflammatory neuropeptides and modulates the immune system by inhibiting monocyte-macrophage functions, B lymphocyte immunoglobulin production, T lymphocyte proliferation and cytokine production (Kolasinski et al, 1992; Pintér et al, 2006). Our data obtained in the same model suggest that tachykinins and CGRP participate in neutrophil accumulation and in the production of the inflammatory cytokine IL-1 β , but do not affect the overall severity of this type of inflammatory reaction. However, in the present model these inflammatory actions of the released pro-inflammatory sensory neuropeptides are counteracted by the anti-inflammatory somatostatin also derived from the C-fibres in response to TRPV1 receptor activation. Since somatostatin and its synthetic agonists have previously been proved to inhibit the outflow of these neuropeptides from isolated rat tracheae, the ability of somatostatin released from sensory nerves to diminish the release of substance P and CGRP might be –at least partially-involved in its inhibitory effect. These data provide the first evidence for a TRPV1 receptor-dependent novel type of counter-regulatory mechanism developing during airway inflammation via the release of somatostatin.

Our results provide the first immunohistochemical and molecular biological evidence for the expression of sst₄ receptors in the mouse lung. In the intact, uninflamed tissue sst₄ is predominantly localized on bronchial epithelial cells, but in the inflamed lung the large number of infiltrating mononuclear cells show markedly increased positivity. No difference was observed, however, between the concentration of sst₄ mRNA in the intact and inflamed lungs despite the immunohistological finding. Since this type of inflammation is an acute pneumonitis which develop within 24 h, it can be assumed that the receptor protein has already been expressed on the surface of the inflammatory cells by the time they infiltrate and not synthesized in the inflamed lung. Receptor upregulation in these mononuclear cells is not

likely in this model. This first functional study with sst₄ receptor gene-deleted mice has clearly revealed that endotoxin-induced airway inflammation determined by myeloperoxidase activity, inflammatory cytokine measurements and histological assessments, as well as consequent bronchial hyperreactivity are enhanced in the lack of the sst₄ receptor compared to their wildtype counterparts.

Somatostatin sst₄ receptor agonists, the heptapeptide TT-232 and the highly selective peptidomimetic agonist J-2156, effectively inhibited airway inflammation and consequent hyperreactivity in both the endotoxin-induced acute and ovalbumine-evoked chronic murine models. These ligands inhibited the cellular phases of inflammation (granulocyte accumulation as determined by the myeloperoxidase activity and production of the inflammatory cytokine IL-1 β) in the endotoxin-induced murine pneumonitis model (Helyes et al, 2006). Since granulocytes themselves have not been shown to express sst₄ receptors, the inhibitory effects of TT-232 and J-2156 on neutrophil/eosinophil are likely to be due to their ability to decrease the release of chemotactic and inflammatory mediators from other cells and nerve terminals. The finding that a single administration of these compounds after the development of the inflammatory reactions also markedly inhibited carbachol-induced bronchoconstriction indicates that they exert direct bronchodilating effect as well. This action might be explained by the localization of sst₄ receptors on bronchial smooth muscle cells, such that their activation inhibits muscarinic receptor-induced contraction. In the ovalbumine-induced chronic inflammation model the anti-inflammatory effects of repeated J-2156 and TT-232 administrations were similar, but the inhibitory action of the latter agonist on airway hyperresponsiveness was much greater. The reason may be that besides sst₄ TT-232 also activates sst₁ receptors and exerts tyrosine kinase inhibitory effect (Helyes et al., 2005). On the basis of the present results, it can be concluded that stable, sst₄-selective agonists could be potential candidates for the development of a completely novel group of anti-inflammatory drugs for the treatment of airway inflammation and also consequent hyperresponsiveness.

SUMMARY OF THE NEW RESULTS

1. Our results provide the first evidence that during endotoxin-induced airway inflammation of the mouse neurogenic inflammatory mediators, such as SP and CGRP, are released from capsaicin-sensitive sensory nerve terminals of the lung, since inactivation of these afferents by RTX pretreatment prevented the LPS-induced increase of these neuropeptides. However, RTX desensitization did not alter the basal concentration of these peptides, which point out that they are also derived from cellular sources. Furthermore, we have also proved with selective receptor antagonists that these neuropeptides play a minor role in the overall severity of the inflammation, NK1 receptors are involved only in increasing granulocyte accumulation. On the contrary inflammatory bronchial hyperreactivity is mediated predominantly by NK2 receptor activation.
2. We have shown a TRPV1 receptor-dependent novel counter-regulatory mechanism in endotoxin-induced airway inflammation, since both the inflammatory reaction and the consequent bronchial hyperresponsiveness were enhanced in TRPV1 gene-deficient mice. We have provided biochemical and functional evidence that somatostatin released via TRPV1 receptor activation mediates these inhibitory effects.
3. The present results provide the first molecular biological and immunohistochemical evidence for the expression of somatostatin sst₄ receptors in the mouse lung, immunopositivity was detected on bronchial epithelial cells and mononuclear cells. Studies with sst₄ receptor gene-deficient mice revealed that the anti-inflammatory and anti-hyperreactivity effects of somatostatin are mediated predominantly via this receptor subtype.
4. Histopathological and functional data have shown that sst₄ receptor agonists, the heptapeptide TT-232 and the highly selective peptidomimetic agonist J-2156, effectively inhibit airway inflammation and consequent hyperreactivity in both the endotoxin-induced acute and the ovalbumine-evoked chronic murine models. Based on these results, they can open novel therapeutic perspectives for the treatment of inflammatory diseases of the lung.

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